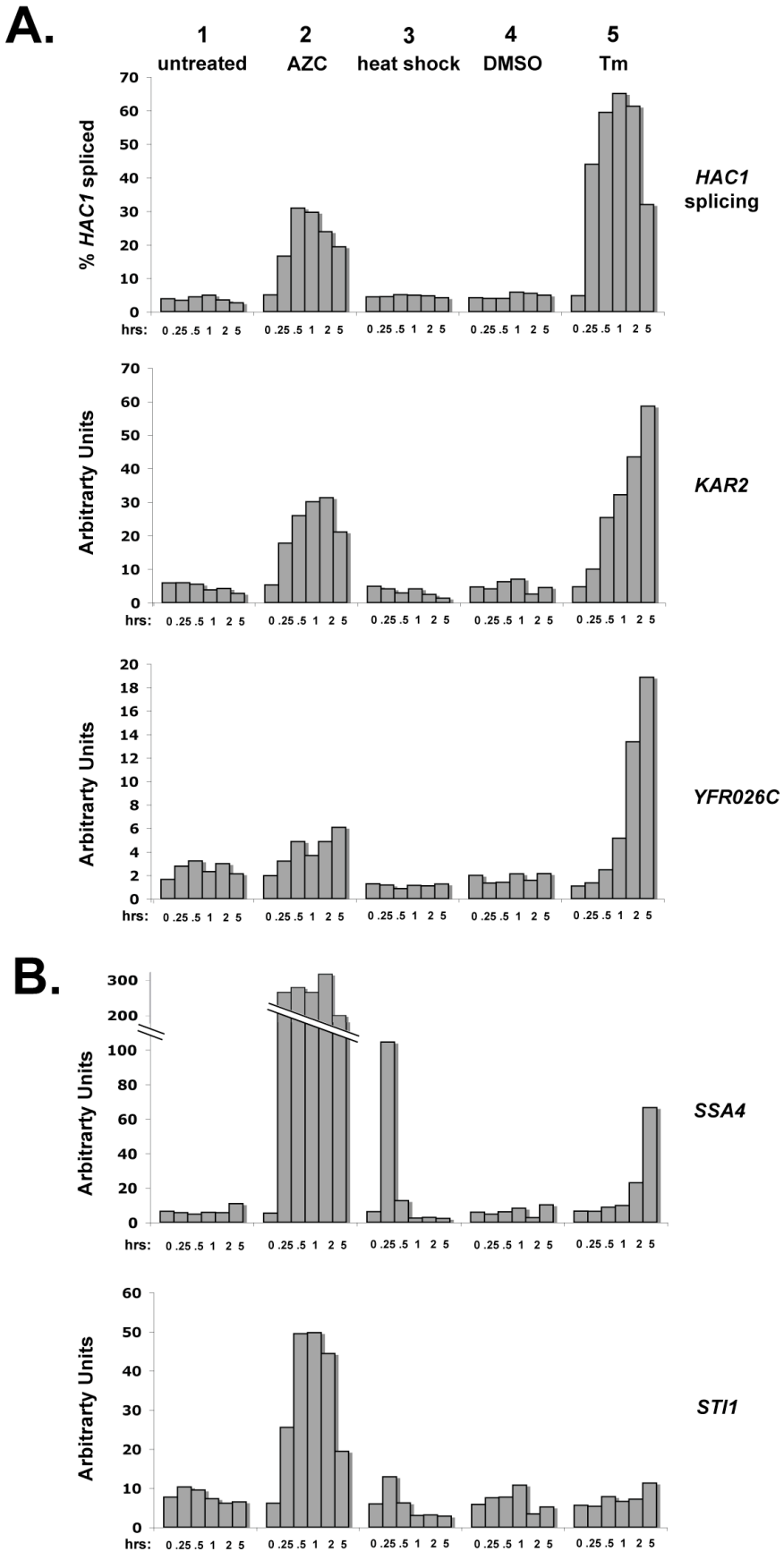
Supplemental Figure S1



Supplemental Figure S1. The transcriptional response to global stresses. Cells were grown to early log phase and treated with various stressors (untreated, AZC, heat shock, DMSO, or Tm; see Supplemental Materials and Methods for details) for 0.25, 0.5, 1, 2, or 5 h before harvesting for RNA processing and Northern blotting. Cultures were harvested for the 0 h time point just prior to addition of drug or temperature shift. Northern blotting was done with the indicated probes to either UPR-L genes (*HAC1* splicing, *KAR2,* and *YFR026C*; A) or cytosolic chaperone genes (*SSA4* and *STI1*; B). Northerns were performed in duplicate and shown is a representative experiment in which quantitated, normalized values, determined as described in Materials and Methods, are graphed as percent of *HAC1* spliced or arbitrary units.

Supplemental Table 1. Genes significantly induced by the expression of *ste6-Q1249X*, but not wild-type *STE6* (see Materials and Methods and Results for description of how these genes were identified).

Supplemental Materials and Methods

Strain and Plasmid Constructions

The *doa10Δ hrd1Δ* strain (SM5360) was constructed by generating a *MAT***a** *doa10::natMX* strain by replacing the *kanMX* cassette of *doa10::kanMX* (Open Biosystems) by recombination with the *natMX* cassette generated from digestion of pAG25 (Goldstein and McCusker, 1999) with *Not*I. This strain was mated to a *MAT*α

hrd1::kanMX strain (Open Biosystems), diploids were sporulated and tetrad dissection yielded a *MAT***a** doa10*::natMX hrd1::kanMX* segregant (SM5360).

Plasmids used in this study are listed in Table 2. Relevant regions of all newly made constructs were verified by DNA sequencing. The *GAL1* promoter was used to induce expression of misfolded proteins. The plasmids pSM1897 (*2μ URA3* P_{GAL1} *STE6-GFP*) and pSM1898 (*2μ URA3* P_{GAL1} *ste6-G38D-GFP*) were generated by homologous recombination of the *GAL1* promoter PCR-amplified from pSM922 (*2μ URA3* P_{GAL1}) into *Swa*I-linearized pSM1493 (*2μ URA3* P_{STE6} *STE6::GFPc*) and pSM1508 (*2μ URA3* P_{STE6} *ste6-G38D::GFPc*) (Huyer et al., 2004a), respectively. This resulted in complete replacement of the *STE6* promoter with the *GAL1* promoter, and galactose-inducible expression was confirmed by fluorescence microscopy and Western blot analysis. To create the L1239X premature truncation allele of Ste6p, pSM2212 (*2μ URA3* P_{GAL1} *ste6-L1239X)* was constructed by PCR amplifying a fragment from the C-terminus of *STE6* using a mutagenic 5′ oligonucleotide containing a stop codon (TAA) at amino acid residue 1239 of Ste6p and recombining this fragment into pSM1897 gapped with *BamH*I. A construct containing the Q1249X truncation, pSM2213 (*2μ URA3* P_{GAL1} *ste6-Q1249X)*, was made in the same manner, but using a 5′ mutagenic oligonucleotide containing a stop codon (TAA) at amino acid residue 1249.

To create a *URA3*-marked, galactose-inducible version of CPY*, pSM2215 (*CEN URA3* P_{GAL1} CPY*-HA) was constructed by subcloning a *Sac*I and *Xho*I fragment containing P_{GAL1} *CPY*-HA* from pES67 (*CEN LEU2* P_{GAL} CPY*-HA; a generous gift from Davis Ng, National University of Singapore) (Spear and Ng, 2003) into the same sites in pRS426 (Sikorski and Hieter, 1989). A high copy version of *pma1-D378S* was

created by PCR amplifying P_{GAL1}-*pma1-D378S* from pRN409U (*CEN URA3* P_{GAL1} *pma1-D378S;* a generous gift from Amy Chang, University of Michigan) (Nakamoto *et al.*, 1998), introducing *Not*I and *Sac*I sites at the 5′ and 3′ ends, respectively. The resulting PCR product was digested with *Not*I and *Sac*I and cloned into the same sites in pRS426 to yield pSM2217 (2μ *URA3* P_{GAL1} *pma1-D378S*).

To assay proteasome activity, we created a low-copy number plasmid that constitutively expressed the N-end rule substrate, ubiquitin-Pro-*LacZ*. *Ub-Pro-LacZ* was PCR amplified from pSM2017 (*2μ LEU2* P_{GAL1} *Ub-Pro-LacZ*; a generous gift from Mark Hochstrasser, Yale University) (DeMarini *et al.*, 1995), adding flanking *Spe*I and *Sac*II sites to the 5′ and 3′ ends, respectively. Following digestion with *Spe*I and *Sac*II, the insert was cloned into the same sites in pRS315 (Sikorski and Hieter, 1989) to generate pMB53 (*CEN LEU2 Ub-Pro-LacZ*). The PGK promoter was PCR-amplified from pSM703 (Huyer *et al.*, 2004b) and recombined into pMB53, gapped using *Sal*I and *Pst*I sites, resulting in pSM2216 (*CEN LEU2* P_{PGK} *Ub-Pro-LacZ).*

Microarray Hybridization and Analysis

RNA from control (empty vector) and experimental (*ste6-Q1249X*, *STE6,* or VHL) samples was processed using the RNA amplification protocol described by Affymetrix (Affymetrix Expression Manual). Briefly, one microgram of total RNA was used to synthesize first strand cDNA using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, California). Following double stranded cDNA synthesis, the product was purified using an Affymetrix sample clean-up column, and biotinylated anti-sense cRNA was generated

through in vitro transcription using T7 RNA Polymerase. Biotinylated cRNA (15 μg) was fragmented at 94°C for 35 minutes (100mM Tris-acetate pH 8.2, 500mM Potassium-acetate, 150mM Magnesium-acetate), and 10 μg of total fragmented cRNA was hybridized to the Affymetrix GeneChip yeast Genome 2.0 array for 16 hours at 45°C with constant rotation (60 rpm). An Affymetrix Fluidics Station 450 was used to remove the non-hybridized target and to incubate with a streptavidin-phycoerythrin conjugate to stain the biotinylated cRNA. The staining was amplified using goat IgG as blocking reagent and biotinilated goat anti-streptavidin antibody, followed by a second staining step with a streptavidin-phycoerythrin conjugate. Fluorescence was detected using the Affymetrix G3000 GeneArray Scanner and image analysis of each GeneChip was done through the GeneChip Operating System 1.4 (GCOS) software from Affymetrix, using the standard default settings. For comparison between different chips, global scaling of all arrays to a target intensity (TGT) of 150 was used.

The quality of the microarray experiment was assessed using the BioConductor packages *affyPLM* and *Affy* for statistical analysis of microarray data (http://www.bioconductor.org). Utilizing *Affy*, the subset of *S. cerevisiae* probe signal values was normalized with the quantile normalization method (Bolstad *et al.*, 2003) and Robust Multi-Array (RMA) expression measures (Irizarry *et al.*, 2003) were obtained for the *S. cerevisiae* probe sets after all probe signal values from CEL files of the chips were background-adjusted. Exploratory data analysis (EDA) was performed with only the *S. cerevisiae* data preprocessed above. Between-treatment and between-replicate variations were examined with the pair-wise MvA plots, in which the base 2 log ratios (M) between two samples are plotted against their averaged base 2 log signals (A). Principal

Component analysis (PCA) was also performed to assess sample variability. Differential gene expression between control and experimental conditions was assessed by statistical linear model analysis using the BioConductor package *limma,* in which an empirical Bayes method is used to moderate the standard errors of the estimated log-fold changes of gene expression, resulting in more stable inference and improved power, especially for experiments with small numbers of microarrays (Smyth, 2004). The moderated t-statistic p-values derived from the *limma* analysis above were further adjusted for multiple testing by Benjamini and Hochberg's method to control false discovery rate (FDR). The FDR cutoff of <1% was used to obtain the lists of differentially expressed genes. All computation was performed under *R* environment (http://www.r-project.org).

Drug and Heat Shock Treatment

To analyze the cellular response to a variety of global stresses, cultures were treated in the following way prior to harvesting for total RNA at the indicated times post-treatment. Control, untreated cells were grown continually at 30°C. Heat shocked cells were grown at room temperature (24°C) to early log phase and then shifted to 36°C at time 0 h. Cells treated with L-azetidine-2-carboxylic acid (AZC; Sigma-Aldrich), DMSO, or Tunicamycin (Tm) were grown at 30°C to early log phase and 10 mM AZC (in dH_2O), 30 μL of DMSO, or 10 μg/ml Tm (in DMSO) were added at time 0 h, respectively.